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Cajal-Retzius Cells Regulate the Radial Glia Phenotype in the Adult and Developing Cerebellum and Alter Granule Cell Migration

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Summary

Studies on the *reeler* mutation have shown that pioneer Cajal-Retzius (CR) cells are involved in neuronal migration in the developing cortex. Here, we use grafting and coculture experiments to investigate the mechanisms by which CR cells govern migration. We show that transplantation of embryonic CR cells, but not other cortical neurons, into adult cerebella induces a transient rejuvenation of host Bergmann glia into a radial glia phenotype. Similarly, CR cells sustain the phenotype of developing radial glia in postnatal cerebellar slices and induce the organization of a glial scaffold inside the CR cell explants. Studies with semipermeable inserts show that these effects are mediated by diffusible signals. We also show that CR cells adjacent to the surface of cerebellar slices reverse the direction of the migration of granule cells. Finally, CR cells from *reeler* mutant embryos elicited similar effects. These observations imply a role for CR cells in the regulation of the radial glia phenotype, a key step for neuronal migration, and suggest that these pioneer neurons may also exert a chemoattractive influence on migrating neurons.

Introduction

Neuronal migration, in which postmitotic neurons migrate from the proliferative zones to the final destination areas, is an essential step in neural development. In particular, directional migration and precise spatiotemporal order of positioning are crucial to the normal cytoarchitectonic organization and pattern of synaptic connections of laminated brain regions such as the cerebellum and the cerebral cortex (reviewed by Hatten, 1993; Rakic et al., 1994). Neuronal migration occurs mainly through specialized glial cells, the radial glia, which provide the adhesive substrate for the translocation of migrating cells and a spatially organized scaffold that defines ordered migratory paths (Ramón y Cajal, 1955; Rakic, 1971, 1972; Edmondson and Hatten, 1987; Hatten, 1990). After migration, radial glial cells are mainly transformed to mature astrocytes and in the cerebellum to Bergmann fibers (Schmechel and Rakic, 1979; Edwards et al., 1990; Misson, 1991; Misson et al., 1991). Moreover, evidence that a radial glia phenotype supports neuronal migration in the adult brain, after neuronal grafting or in adult songbirds, has emphasized the relevance of radial glia for migration (Alvarez-Buylla and Nottebohm, 1988; Sotelo and Alvarado-Mallart, 1988; Sotelo et al., 1994). In addition, neurons migrating tangentially may glide along other substrates, such as axon fascicles (Bourrat and Sotelo, 1988; O'Rourke et al., 1992, 1995; Reid et al., 1995).

Radial glia are among the first cells to differentiate. They are characterized by typical bipolar shapes and the expression of specific markers, including nestin, brain-lipid-binding protein, and the antigens recognized by the RC1 and RC2 Mabs and the D4 antisera (Hockfield and McKay, 1985; Misson et al., 1988, 1991; Edwards et al., 1990; Gadisseux et al., 1992; Cameron and Rakic, 1994; Feng et al., 1994; Feng and Heintz, 1995). Although radial glia has a crucial role in development, the mechanisms regulating its identity and function are not known. Recent studies showing that mature astrocytes are transformed into a juvenile radial glia phenotype in response to diffusible factors from embryonic neurons (Sotelo et al., 1994; Hunter and Hatten, 1995a) suggest that radial glial cell specification and differentiation are controlled by soluble signals, similar to what has been shown for the lineage and differentiation of oligodendrocytes and Schwann cells (e.g., Raff et al., 1988; Gard et al., 1995).

The histogenesis of the cerebral cortex follows an "inside-out" sequence of neuronal positioning and maturation, in which migrating neurons bypass earlier neurons and settle just below the marginal zone layer I, thus forming the typical layered organization of the cortex (Angevine and Sidman, 1961; Rakic, 1990; Bayer and Altman, 1991). Together with subplate neurons (Allendoerfer and Shatz, 1994), the Cajal-Retzius (CR) cells are the earliest-generated cortical neurons and the first to mature. These transient neurons lie in a characteristic, subpial location in layer I, and most of them disappear by cell death at postnatal ages (Marín-Padilla, 1971, 1984, 1988; Edmunds and Parnavelas, 1982; Derer and Derer, 1990, 1992; Del Río et al., 1995). Because of their strategic location in layer I, where all migrating neurons migrate, and the coincidence of their life span with the period of cortical migration, it has been proposed that CR cells may have a role in migration (Marín-Padilla, 1988; Del Río et al., 1995). Recent findings in the *reeler* mutant mouse, a genetic abnormality with altered migration in many brain regions including the neocortex and cerebellum (Caviness and Sidman, 1973; Mariani et al., 1977; Caviness, 1982), show that the defective gene—*reelin*—is expressed by CR cells (Goffinet, 1995; D'Arcangelo et al., 1995; Hirotsune et al., 1995). Furthermore, inhibition of Reelin with neutralizing antibodies disrupts the histotypic organization of reaggregation cultures (Ogawa et al., 1995).

The mechanisms by which CR cells are involved in migration are not known. CR cells might act either on the functional differentiation of radial glia or on the attraction of migrating cells. These processes could be

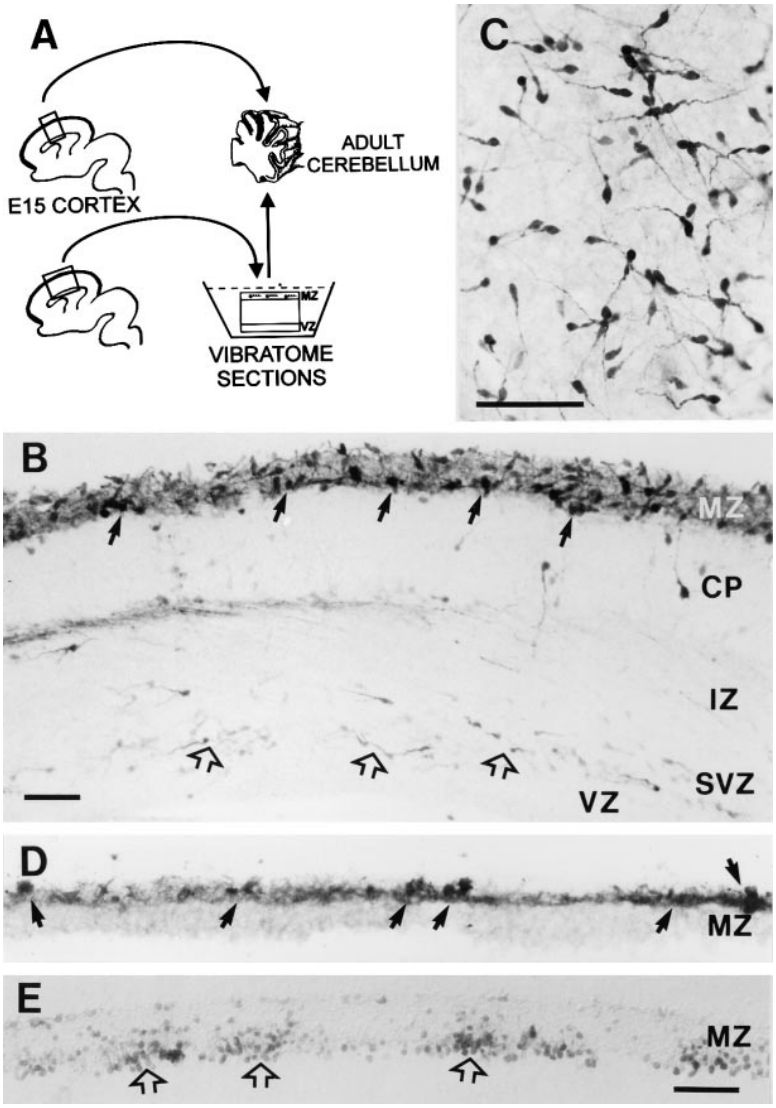

Figure 1. Schematic Drawing and Composition of Donor Tissues for the Grafting Experiments

(A) Diagram of the transplantation procedures. In some experiments (top), pieces of tissue from E15 embryos containing all the layers of the cerebral cortex were grafted into adult host cerebella. In other experiments (bottom), pieces from E15 neocortex were tangentially cut in a vibratome, and the slices containing the marginal zone layer I (MZ) were used for transplantation. VZ, ventricular zone.

(B) Frontal section from the neocortex of an E15 embryo immunoreacted with calretinin antibodies illustrating the cell composition and layering of the cortex at the age at which the vibratome slices were taken. Calretinin+ CR cells (arrows) are densely packed in the marginal zone layer I (MZ). Immunoreactive neurons are very scarce in the remaining cortical layers, including the cortical plate (CP), intermediate zone (IZ), and ventricular zone (VZ). In the subventricular zone (SVZ), many immunoreactive, small cells can be seen (open arrows).

(C) Tangential view of layer I at P2 in a thick neocortical slice immunoreacted for calretinin, illustrating CR cells with typical pearl-shaped to ovoid perikarya and a single thick dendrite.

(D and E) Transverse cryostat sections (20 μm thick) of tangential vibratome slices through the marginal zone obtained as shown in (A), illustrating the cellular composition of the vibratome slices used for transplantation and culture experiments. The section in (D) was immunoreacted for calretinin and shows numerous CR cells (arrows). The section in (E) was stained with cresyl violet and shows that the vibratome slice contains, in addition to cell components of the marginal zone (MZ), some clusters of neurons from the upper cortical plate (open arrows) but not neurons from the deep cortical plate, the subplate, the intermediate zone, or the ventricular zone. Scale bars = 100 μm, (B) and (C); and 50 μm, (D) and (E).

mediated via soluble factors or by direct cell–cell contact since the end feet of radial glia terminate in layer I. Here, we grafted embryonic CR cells into adult cerebella and found that CR cells, but not other embryonic cortical cells, induced a transformation of adult Bergmann glia into a radial glia phenotype. Moreover, coculture experiments with semipermeable inserts provided evidence that radial glia-inductive signals released by CR cells are soluble. Last, we show that CR cells dramatically altered the migration pattern of cerebellar granule cells by shifting the direction of granule cell migration. These data indicate that CR cells play a major role in neuronal cell migration by regulating the identity of radial glia and the movement of migrating neurons.

Results

Induction of a Radial Glia Phenotype in the Adult Cerebellum by Embryonic Cortical Neurons

Embryonic Purkinje cells grafted into the adult cerebellum induce the reexpression of radial glia antigenic markers in Bergmann astrocytes (Sotelo et al., 1994). To determine whether embryonic cells from the neocortex could also elicit such rejuvenation of Bergmann glia, blocks of E15–E16 neocortex were transplanted into the cerebella of adult mice (n = 4; Figures 1A and 2). Seven to ten days after grafting (DAG), the transplants were large and located between the cerebellar folia (Figure 2A). Sections immunostained with the RC2 monoclonal antibody, which recognizes an epitope present in developing radial glia but not in mature astrocytes or Bergmann glia (Edwards et al., 1990; Misson, 1991; Hunter and Hatten, 1995a), revealed palisades of RC2+ cells in the host tissue surrounding the graft (Figures 2A and 2C). RC2+ palisades had a patchy distribution in the molecular layer of the host cerebella, occurring only in glial cells that displayed the distinctive morphology of Bergmann glia (Figure 2C). Parallel sections immunostained with antibodies against calretinin, a CR cell marker (Figures 1B and 1C; Soriano et al., 1994; Del Río et al., 1995), disclosed clusters of large labeled neurons with elongated cell bodies and a thick, primary dendrite

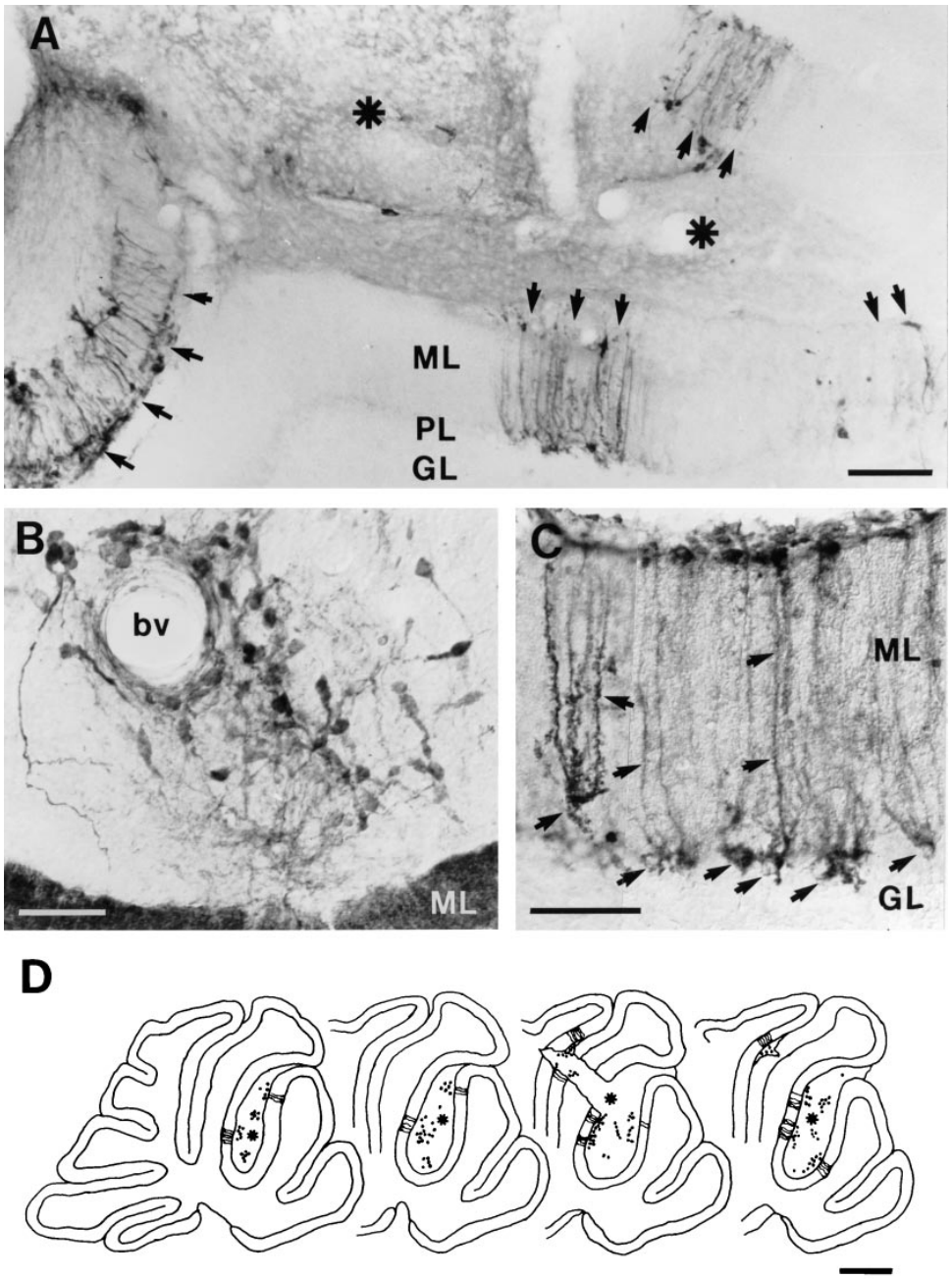

Figure 2. RC2 Induction in Adult Cerebella 7 Days after Transplantation of E15 Neocortex

(A) Sagittal cerebellar section immunolabeled with the RC2 antibody shows the allocation of the graft (asterisks) within several cerebellar folia and the patchy induction (arrows) of RC2 immunoreactivity in the Bergmann glia of the host cerebellum. ML, molecular layer; PL, Purkinje cell layer; and GL, granule cell layer.

(B) Photomicrograph from a graft immunostained with calretinin antibodies illustrating numerous CR cells inside the cortical graft grouped around a blood vessel (bv). Note the intense calretinin staining of the host parallel fibers in the molecular layer (ML).

(C) RC2 immunostaining of host glial cells exhibit the typical morphology of Bergmann glia (arrows). RC2+ cell bodies are located at the granule cell layer–molecular layer (GL–ML) interface (arrows) and have several varicose processes (arrows) that ascend perpendicularly in the molecular layer.

(D) Camera-lucida reconstruction illustrates the extent of RC2 immunostaining in sagittal sections of an adult host cerebellum after grafting pieces of E15 neocortex (asterisks). Note the relationship between the distribution of CR cells identified by calretinin immunocytochemistry (dots) and RC2+ Bergmann fibers. Mediolateral sections spaced 250–350 μm. Scale bars = 100 μm, (A); 50 μm, (B) and (C); and 500 μm, (D).

(Figure 2B). These features are distinctive of murine CR cells (Figure 1C), indicating that these neurons survive grafting. Serial section reconstructions showed that RC2 reexpression was correlated with the clusters of calretinin+ CR cells (Figure 2D). These results indicate that embryonic neurons from neocortex induce a rejuvenation of adult Bergmann glia and suggest that the factors regulating the radial glia phenotype are common to different brain areas.

CR Cells Are Responsible for the Induction of a Radial Glia Phenotype in the Adult Cerebellum

The developing E15–E16 neocortex contains distinct cell populations such as glial cells, progenitors, migrating neurons, and subsets of postmitotic neurons, including CR cells (Figure 1B). To establish whether CR cells were responsible for the reexpression of radial glia antigenic markers, we developed a procedure to enrich CR cells by separating layer I from the remaining cortex (Figure 1A). Taking advantage of the superficial location of CR cells in the marginal zone layer I (Figure 1B), we obtained tangential vibratome sections (60–80 μm thick) of flattened cortices, which contained almost exclusively tissue from layer I. These vibratome sections were rich in CR cells, as identified with calretinin immunostaining (Figures 1D and 1E). Exceptionally, a few immature neurons from the uppermost cortical plate were also attached, but cells from the remaining cortical layers, i.e. the cortical plate, subplate, and the intermediate, ventricular, and subventricular zones, were lacking (Figure 1E).

Transplantation of the CR cell–enriched slices into adult cerebella resulted in smaller grafts at 3–10 DAG (Figure 3) compared to those described above, as expected from implants containing postmitotic CR cells, but neither progenitors nor migrating neurons. Following calretinin immunostaining, CR cells were seen inside the grafts, although the dense immunolabeling of the host cerebellum hindered their identification. We thus transplanted CR cell–enriched slices from transgenic mouse embryos carrying the promoter region of the Neuronal Specific Enolase (NSE) gene linked to the *lacZ* reporter gene, which allowed us to identify grafted CR cells by Xgal-histochemistry. Again, most grafted CR cells were located on the surface of the cerebellum (Figure 3D) and some along the pipette track. Grafting of CR cell–enriched slices gave rise to a strong induction of RC2 immunoreactivity in large palisades of Bergmann glia (Figure 3A) facing clusters of grafted Xgal+ CR cells (Figures 3D and 3F). The cerebellar areas showing induction of the RC2 epitope were larger and denser (Figure 3F) than those seen when all of the layers of the developing cortex were grafted (Figures 2A and 2D).

To investigate whether cortical cells other than CR cells might induce RC2 reexpression, we transplanted vibratome slices containing only the ventricular and subventricular zones (enriched in neuronal and glial progenitors and in radial glia) and slices containing all cortical layers except layer I, thus containing postmitotic neurons from the subplate and cortical plate, progenitors, and migrating cells. After grafting both preparations, RC2 immunostaining was prominent inside the transplants, labeling clusters of small, round cells, which were most probably undifferentiated radial glia (Figure 3C). In contrast, bipolar differentiated radial glia were extremely rare inside the grafts, and we did not observe induction of RC2 immunostaining in cerebellar Bergmann glia in any of the animals transplanted ($n = 7$) at any of the survival times tested (4–17 DAG). We thus conclude that CR cells but not cortical precursors or migrating neurons, radial glia, or other populations of postmitotic cortical neurons can elicit the transformation of adult Bergmann glia to a RC2+ radial glia phenotype.

RC2 Reexpression Occurs Transiently in Host Bergmann Glia

Following transplantation of either the complete cortex or CR cell–enriched slices, most RC2+ glial cells had their cell bodies at the level of the Purkinje cell layer. They exhibited vertical branches with leaf-like excrescences and terminated in end feet near the pial surface (Figures 2C and 3B), as adult Bergmann fibers (Altman, 1982). Only very exceptionally were other glial cells RC2+. These glial cells were always present in the molecular layer and had bipolar shapes (Figure 3E). To exclude the possibility that RC2+ glial cells had differentiated from grafted glial cells, we transplanted CR cell–enriched slices into the cerebellum of adult mice ($n = 4$) of the Krox20–*lacZ*14 transgenic line. This line was generated using a hybrid Krox20–*lacZ* gene construct, but it shows a very specific and ectopic expression of βgal in developing and mature Bergmann glia (Figure 4A; see Sotelo et al., 1994). Double immunolabeling showed that virtually all RC2+ processes were also βgal-immunoreactive (Figures 4B and 4C), demonstrating that RC2 reexpression occurred in adult Bergmann glia from the host cerebella.

After transplantation of CR cell–enriched slices, RC2 immunostaining was very weak at 4 DAG ($n = 5$), maximal after 6–12 DAG ($n = 29$), and decreased at 15 DAG ($n = 4$). No RC2+ staining was detected at 20 DAG ($n = 5$). At short (4 DAG) and long (15 DAG) survival times, the cerebellar areas exhibiting RC2 reexpression were smaller than at 6–12 DAG, and the immunolabeling was mainly confined to the cell bodies and primary branches of Bergmann glia (not shown), in contrast to the complete staining observed at 6–12 DAG (Figure 3B). Thus, grafting of CR cells led to a transient rejuvenation of host Bergmann glia in adult cerebellum. The timing of RC2 reexpression might correlate with the fate of grafted CR cells since from 12 DAG on, these neurons were progressively less conspicuous, suggesting that they disappeared by cell death as in the cerebral cortex (Del Río et al., 1995).

CR Cells Promote the Organization of a Glial Scaffold in Organotypic Cerebellar Cultures

To determine whether CR cells may also affect developing glial cells, CR cell–enriched slices were cultured directly in contact with the surface of organotypic cultures from P6–P8 cerebella (Figure 5A), a stage at which cerebellar radial glia had ceased to express the RC2

epitope (Edwards et al., 1990; Misson, 1991). Immunostaining of CR cell–enriched slices with calretinin antibodies after 7–14 days in vitro (DIV) showed that CR cells displayed healthy shapes (Figure 6H), indicating that these neurons survived under the present culture conditions. After 7–14 DIV, RC2+ glial cells were detected only in the area of the cerebellar slices proximal to the CR cell explants. Most RC2+ cells bore thin processes in the molecular layer and exhibited the distinctive morpholgy of immature Bergmann glia, indicating that CR cells could also sustain the phenotype of immature radial glia in the cerebellum. In addition, in all cocultures, we observed numerous, extremely long RC2+ processes (>500 μm) and RC2+ glial cells that penetrated deeply into the CR cell–enriched explants (Table 1), suggesting that the latter may induce ingrowth of radial glial processes and cells. RC2 immunoreactivity was absent from single cerebellar cultures and from cerebellar slices cocultured with explants containing the ventricular zone, the subplate, or the cortical subplate. Some of such cortical explants (6 of 15) displayed RC2+ glial cells, indicating that cortical glial cells may retain their radial glia phenotype under these in vitro conditions (Table 1).

To examine whether CR cells might exert a morphogenetic influence on cerebellar glia, we obtained cerebellar slices from the Krox20–*lacZ*14 line and cocultured them with CR cell–enriched explants. Cortical explants containing the ventricular zone or layers other than layer I were used as controls in a triple confrontation assay (Figure 5A). After 7–14 DIV, cultures were stained for βgal activity to reveal the distribution of cerebellar Bergmann glia. In the CR cell–enriched explants, there was a massive ingrowth of βgal+ Bergmann glia (Figure 6A; Table 1). Their protoplasmic processes formed a dense, complex scaffold, penetrating 400–600 μm into the explant, with most of the processes oriented radially, i.e., perpendicular to the granule cell layer (Figure 6B). These radial glial cell processes were in close relationship with clusters of CR cells in the neocortical slice (Figure 6C). In contrast, most cerebellar slices cocultured with control cortical explants exhibited a complete absence of glial cell ingrowth (Figure 6A), although in some cultures, a few Xgal+ glial processes penetrated 100–200 μm into the control explants (Table 1). These findings indicate that CR cells exert a growth-promoting, morphogenetic influence on cerebellar radial glia.

Induction of RC2 Expression by CR Cells Is Mediated by Diffusible Factors

To determine whether the induction of radial glia by CR cells could be mediated by soluble signals, postnatal cerebellar slices were overlaid by CR cell–enriched explants (or other neocortical tissue), separated by a semipermeable membrane (Figure 5A). Following 7–10 DIV, cerebellar slices overlaid with CR cell explants exhibited a dramatic induction of RC2 immunostaining with a high density of RC2+ cells (Table 1; Figures 5B–5D). The induction of RC2 immunostaining was stronger than in the culture experiments described above or after CR cell grafting into adult cerebella (compare with Figures 2 and 3). Moreover, RC2+ cells were not restricted to the Purkinje cell and molecular layers, where Bergmann glial cells are located, but were distributed throughout all cerebellar layers. Some RC2+ cells exhibited round perikarya, varicose processes and shapes close to those of developing Bergmann glia (Figure 5D). However, most RC2+ glial cells had elongated bipolar shapes with two thick processes arising from opposite sides of the cell body (Figure 5C), which is similar to the morphology of immature radial glia (Rakic, 1971; Edwards et al., 1990; Misson, 1991). Occasionally, the long processes of these cells formed bundles.

In contrast, cerebellar explants cocultured with other cortical tissue containing the proliferative layers or postmitotic neurons from deep cortical layers, as well as control slices cultured with semipermeable membranes alone, did not show RC2 immunostaining (Table 1). These findings indicate that CR cells induce a radial glia phenotype via diffusible factors. Moreover, the obtained dramatic induction of radial glia suggests that the degree of induction depends on the availability of such diffusible signals.

CR Cells Perturb the Migration of Cerebellar Granule Cells in Culture

In the early postnatal cerebellum, progenitors for granule cells and for molecular layer interneurons divide, and the daughter cells migrate to their respective final destinations (Miale and Sidman, 1961; Gao et al., 1992; Zhang and Goldman, 1996). To determine whether CR cells can influence the pattern of migration of these late dividing neurons, cerebellar slices (P5–P8) taken from the β2nZ3′1 transgenic mouse line were cocultured in contact with CR cell–enriched explants. The β2nZ3′1 transgenic line, generated using a β2-microglobulin/*lacZ* construct (Cohen-Tannoudji et al., 1992; Soriano et al., 1995), was used because it shows ectopic expression of βgal in postmitotic granule cells and in molecular layer interneurons but not in Purkinje cells or glial cells (Jankovski et al., 1996). After 7–14 DIV, the cocultures were stained with Xgal and immunolabeled with antibodies to either calretinin to identify CR cells, calbindin 28kDa to label Purkinje cells, or parvalbumin, which in addition to Purkinje cells also labels the molecular layer interneurons (Celio, 1990; Jankovski et al., 1996). While the cytoarchitecture was well preserved in the cerebellar slices, there was a marked alteration in the distribution of granule cells (Figure 6D; Table 2). In the areas of direct contact between the two explants, a large number of neurons expressing βgal activity provided with small nuclei (granule cells) penetrated up to 800–1000 μm into the neocortical explants (Figure 6E). In addition, slices immunoreacted for βgal and parvalbumin showed that some larger neurons, which had migrated into the CR cell explants (11%; N = 551 cells, 4 cultures), were double labeled, thus corresponding to basket and stellate cells (not shown). Granule cells, although spread throughout the CR cell explants, were mostly concentrated in a narrow band 400–500 μm from the cerebellar surface, mimicking an internal granule cell layer in the neocortical tissue (Figure 6E). In other slices, the cerebellar neurons within the CR cell explants were arranged in clusters, which followed the distribution of CR cells,

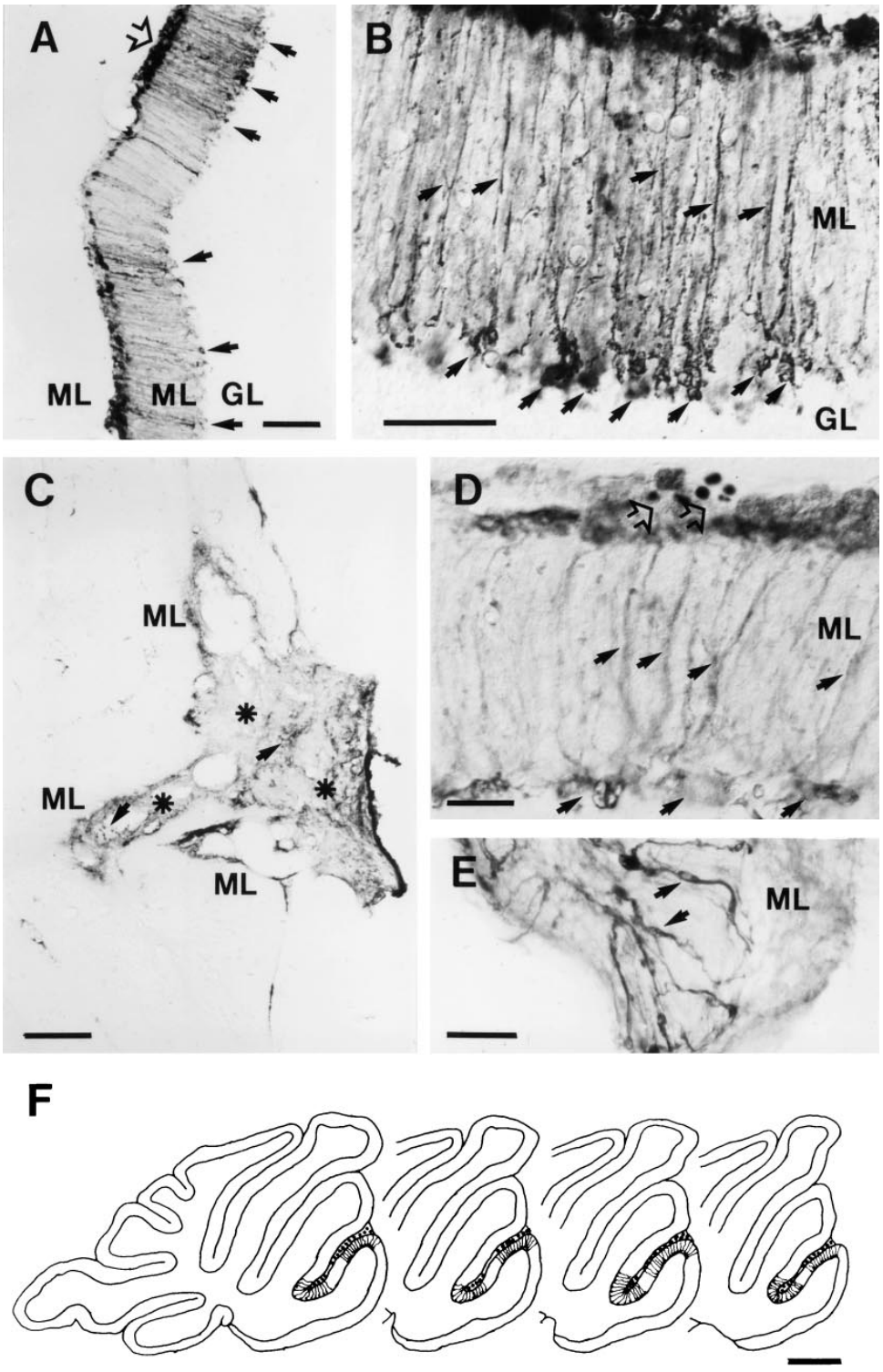

Figure 3. Induction of RC2 Immunostaining in Adult Cerebella 7–10 Days after Transplantation of CR Cell-Enriched Slices. Abbreviations as in Figure 2

(A) Photomicrograph illustrating large palisades of intense RC2-immunoreactive Bergmann glia (arrows) at 7 DAG.

(B) High magnification view of the palisade labeled by open arrow in (A). Note the dense RC2 immunolabeling of Bergmann glial cells and processes (arrows) spanning the molecular layer (ML).

(C) RC2 immunostaining after transplantation of a control vibratome slice containing the ventricular and subventricular zones (enriched in radial glia and cortical progenitors) from E15 embryos (7 DAG). Some clusters of RC2+ cells (arrows) occur inside the cortical graft (asterisks). However, no RC2 immunostaining is observed in the host cerebellum (to the left).

(D) Section from a cerebellum grafted with a CR cell–enriched slice taken from an NSE–*lacZ* transgenic embryo (7 DAG). The section has

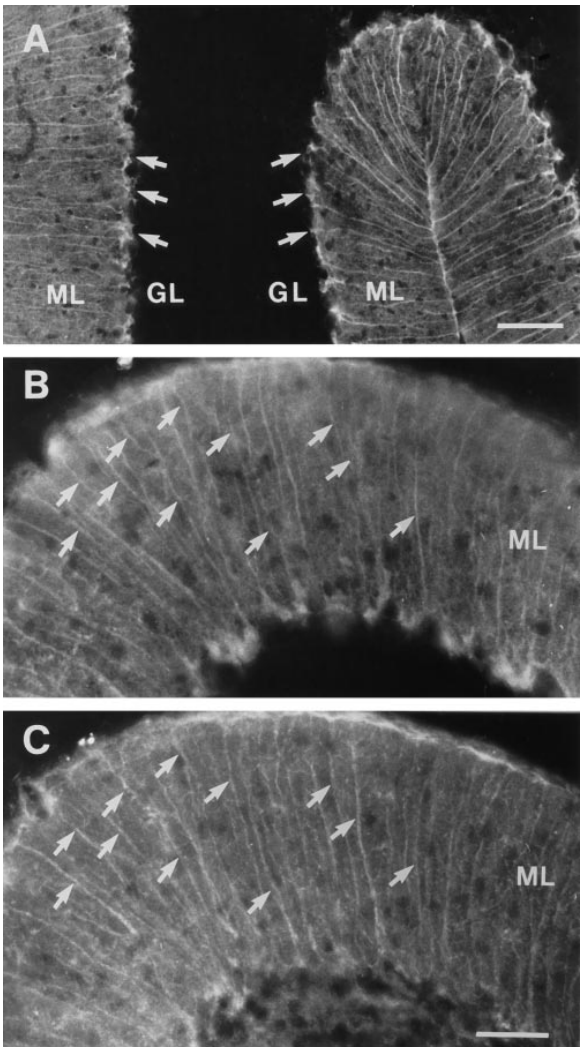

Figure 4. Induction of RC2 Immunostaining in Host Bergmann Glia after Transplantation of CR Cell–Enriched Slices in Krox20–*lacZ*14 Transgenic Mice (7 DAG). Abbreviations as in Figure 2

(A) Fluorescence microphotograph shows the distribution of βgal-immunoreactive elements in the adult cerebellum of a Krox20–*lacZ*14 transgenic mouse. βgal immunolabeling occurs exclusively in Bergmann glial cells and fibers (arrows).

(B and C) Pair of fluorescence photomicrographs of a section double immunolabeled with RC2 (C) and βgal (B) antibodies, after transplantation of a CR cell–enriched slice (7 DAG) in an adult Krox20–*lacZ*14 cerebellum. Glial fibers exhibiting RC2 immunostaining (arrows in [C]) are also βgal immunoreactive (arrows in [B]), demonstrating that RC2 induction occurs in host Bergmann glia. Scale bars = 100 μm, (A); 50 μm, (B) and (C).

as visualized with calretinin immunostaining (Figures 6G and 6I).

The area of the cerebellar slices that was in contact with control cortical tissue showed normal cytoarchitectonics. In contrast to the alterations reported above, very few βgal+ granule cells were seen within the cortical explant, where they penetrated only 100–200 μm (Figure 6F; Table 2). These results show that CR cells dramatically alter the normal pattern of granule cell migration, by shifting the direction of migration followed by postmitotic granule cells.

CR Cells of *reeler* Mutant Embryos Elicit Similar Effects in Radial Glia and Granule Cell Migration

To determine whether Reelin, an extracellular matrix protein (D'Arcangelo et al., 1997), was involved in the induction of radial glia, cerebellar slices were cocultured between semipermeable membranes (Figure 5A) with CR cells from *reeler* embryos. Such cerebellar cultures exhibited an induction of bipolar RC2+ radial glial cells, as strong as that elicited by CR cells from heterozygous or wild-type embryos (Figure 5E; Table 1). These findings suggest that Reelin is not essential for the induction of radial glia by CR cells. Similarly, when β2nZ3′1 cerebellar slices were cocultured with *reeler* CR cells, substantial numbers of cerebellar granule cells had penetrated the *reeler* neocortical tissue for long distances (five of seven). However, additional studies and quantitative experimental paradigms are needed to ascertain whether there are significant differences in the numbers of ectopic granule cells and in the depth of their reverse migration.

Discussion

Here, we used antibody marker expression, heterotopic grafting, and coculture experiments to gain insight into the cellular interactions that regulate the radial glia cell identity. We provide evidence that CR cells, but not other cortical cells, induce the transformation of Bergmann glia into a radial glia phenotype in the adult and postnatal cerebellum, and that this transformation is likely to be mediated by diffusible factors released by CR cells. Moreover, we found that CR cells have a pronounced effect on the normal migration pattern of cerebellar granule cells.

The Radial Glia–Astrocyte Pathway Is Bidirectional and Regulated by Soluble Signals Common to Different Brain Regions

Neuronal cell migration requires the cooperative interaction of adhesion and recognition molecules expressed by migrating neurons, radial glial cells, and postmigratory maturing neurons. Thus, several specific molecules,

been developed with Xgal and then immunoreacted with RC2 antibodies. Xgal-positive CR cells (open arrows) appear located at the cerebellar fissure, just above the sites of Bergmann glia RC2 reexpression (arrows).

(E) RC2-immunoreacted section shows RC2+ cells (arrows) in the molecular layer (ML), with bipolar morphology distinct to that of Bergmann fibers. Such bipolar RC2-immunoreactive cells were observed only occasionally.

(F) Camera lucida reconstruction illustrates the extent of RC2 reexpression in representative sagittal sections (spaced 250–350 μm) from an adult cerebellum grafted with a CR cell–enriched slice from an NSE–*lacZ* embryo. Note the correlation between the distribution of CR cells (dots), identified after Xgal staining, and clusters of RC2+ Bergmann glia.

Scale bars = 100 μm, (A) and (C); 50 μm, (B) and (E); 25 μm, (D); and 500 μm, (F).

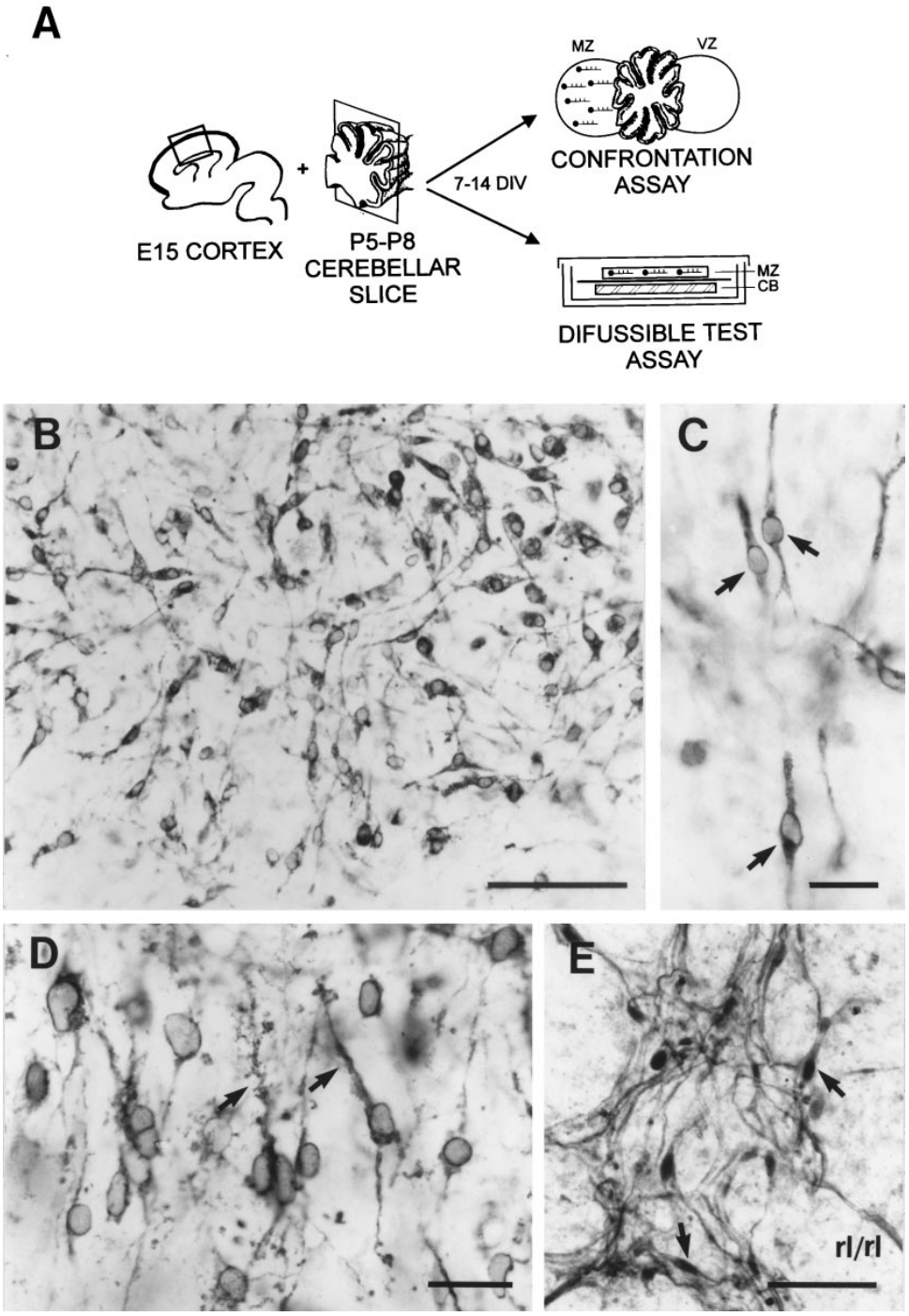

Figure 5. Induction of RC2 Immunolabeling in Postnatal Cerebellar Slices Cocultured with CR cells. Abbreviations as in Figure 2

(A) Schematic representation of the different coculture approaches used. Cerebellar postnatal slices were cocultured with CR cell–enriched explants (MZ) and ventricular zone–enriched explants (VZ) positioned at opposite sites at the surface of the cerebellar slices (contact confrontation assay) or above a semipermeable membrane, which was held onto the cerebellar (CB) slice (diffusible test assay).

(B–E) These micrographs illustrate results obtained with the diffusible test assay using CR cell–enriched slices ([B–D], wild type; [E], homozygous *reeler*).

(B) Dramatic induction of RC2 immunostaining in many cerebellar glial cells throughout the cerebellar slice. Most RC2+ glial cells are bipolar and appear to be randomly oriented.

(C and D) High power photomicrographs illustrate examples of RC2+ cells in cerebellar slices. While some RC2+ cells (D) located in the Purkinje cell layer exhibit a radial orientation and varicose processes (arrows) that may correspond to that of immature Bergmann glia, other cells show very immature bipolar shapes (arrows in [C]) with two thick processes arising from opposite sides of the cell bodies.

(E) Induction of RC2 immunostaining in a cerebellar slice cocultured with a CR cell–enriched slide from a *reeler* mouse embryo. Note numerous RC2+ glial cells displaying bipolar shapes (arrows) and some fasciculation of the processes. Scale bars = 100 μm, (B) and (E); and 25 μm, (C) and (D).

including PSA-NCAM, astrotactin, Reelin (Fishell and Hatton, 1991; Cremer et al., 1994; Ono et al., 1994; Goffinet, 1995; D'Arcangelo et al., 1995, 1997; Hirotsune et al., 1995; Ogawa et al., 1995; Hu et al., 1996; Zheng et al., 1996), and the proteins recognized by the D4 and NJPA1 antibodies (Anton et al., 1996), have a critical role in neuronal migration. A key step for neuronal migration is the specification and differentiation of radial glia. Two recent studies have shown that the transformation of radial glia into mature astrocytes, which occurs at the end of the migration period (Schmechel and Rakic, 1979; Misson et al., 1988, 1991; Edwards et al., 1990), is a bidirectional process mediated by soluble signals. Grafted embryonic Purkinje cells induce a rejuvenation of adult host Bergmann glia, which supports the migration of transplanted Purkinje cells (Sotelo et al., 1994), and cultured dissociated astrocytes from postnatal cortex are transformed into a radial glia phenotype by diffusible factors released by embryonic neocortical cells (Hunter and Hatten, 1995a). The present study gives further support to the notion that, in vitro and in vivo, mature astrocytes acquire a radial glia phenotype in response to appropriate factors and that these factors are most likely diffusible signals. Finally, the heterotopic grafting and coculture experiments showing that cerebellar glial cells respond to factors of cortical origin suggest that these signals are common to both brain regions.

The identity of radial glia–inducing factors and their mechanisms of action are at present unknown. In our grafting experiments in adult mice and when neocortical and postnatal cerebellar explants were cocultured side by side, only the Bergmann glia expresses radial glia antigenic markers, although it does not adopt the bipolar morphology of immature radial glia. In contrast, a dramatic transformation of Bergmann glia and other cerebellar astrocytes to bipolar radial glia occurs when postnatal cerebellar slices are placed underneath neocortical explants but separated by a semipermeable membrane. This suggests that the degree of glial transformation depends on the accessibility and/or concentration of the inducing signals as well as on age-dependent properties of astrocytes. Thus, the present experiments support the contention that the radial glia–astrocyte pathway is bidirectional and that it is mediated by diffusible signals common to different brain areas. However, the ability of adult astrocytes to undergo such transformation may be restricted to certain glial lineages, such as Bergmann glial cells, and to maturational stages (Figure 7A).

CR Cells Regulate the Phenotype of Radial Glia

The present transplantation, coculture, and semipermeable membrane experiments show that radial glia–inducing signals are released specifically from the marginal zone layer I but not from other cortical layers containing radial glial cells, progenitors, or migrating and maturing neurons. Although layer I contains a small subset of γ-aminobutyric acid (GABA)ergic neurons (Cobas et al., 1991; Del Río et al., 1992), a role for such neurons in the regulation of radial glia cell identity seems unlikely since these interneurons are generated sparsely throughout the period of corticogenesis (E11–E17 in the mouse; Fairén et al., 1986), after the radial glia develops, and so few such neurons are in layer I at E15. In contrast, the role of CR cells in migration is supported by: (i) the constant correlation between the survival and location of CR cells and effects on radial glia and migration (here); (ii) their early generation at the beginning of corticogenesis and their life span coincident with the period of cortical migration (Derer and Derer, 1990; Del Río et al., 1995); (iii) the time course analysis showing that radial glia–inducing signals are strong at E14, decrease by E19, and are absent from P6 onward (Hunter and Hatten, 1995a), correlating with the progressive dilution of CR cells in the developing cortex and their disappearance by cell death by P5–P8 (Derer and Derer, 1990; Del Río et al., 1995); and (iv) the gene defective in *reeler* mice is expressed by CR cells (D'Arcangelo et al., 1995; Hirotsune et al., 1995; Ogawa et al., 1995). Thus, all available evidence indicates that CR cells are the neurons responsible for the effects on radial glia and migration reported here.

The present study indicating that CR cells release diffusible factors that regulate the radial glia cell identity and phenotype predicts that in their absence, radial glia would be transformed into mature astrocytes. This is indeed supported by complementary experiments in which the ablation of CR cells in the cortex of newborn mice leads to the opposite effect: loss of radial glia and transformation to astrocytes, accompanied by arrest of migration (H. Supèr, J. A. D. R., A. Martínez, and E. S., unpublished data). Furthermore, our experiments with the Krox20–*lacZ*14 transgenic line show that CR cell–enriched explants induce a dramatic ingrowth of developing Bergmann glia within the explants (Figure 7B). Thus, in addition to regulating the radial glia cell identity, CR cells might exert a growth-promoting, morphogenetic effect on radial glia. We do not know whether CR cells have a similar inductive effect in the developing cortex in vivo, although their strategic location in layer I is especially well suited to exerting such a role and to inducing the organization of the ordered arrays of radial glia terminating in layer I.

The molecular weight of radial glia–inducing signals has been estimated to be ≈55 kDa (Hunter and Hatten, 1995a). Reelin, although a large extracellular matrix protein (D'Arcangelo et al., 1997), could theoretically be at the origin of this uncharacterized active factor, provided that a posttranslational processing occurs. Nevertheless, the present data showing that CR cells from *reeler* embryos also induce a radial glia phenotype, together with the persistence of radial glia in *reeler* mutant mice (Pinto-Lord et al., 1982), indicate that Reelin is unlikely to be the unique soluble factor regulating radial glia cell identity. The radial glia of *reeler* mice, however, shows morphological abnormalities, and it is transformed into mature astrocytes earlier than in normal animals (Derer, 1979; Hunter and Hatten, 1995b). This suggests that Reelin may participate in the regulation of the radial glia–astrocyte pathway, perhaps by facilitating the action of soluble factors as other extracellular matrix proteins do for some growth factors (e.g., Lander, 1993; Schlessinger et al., 1995).

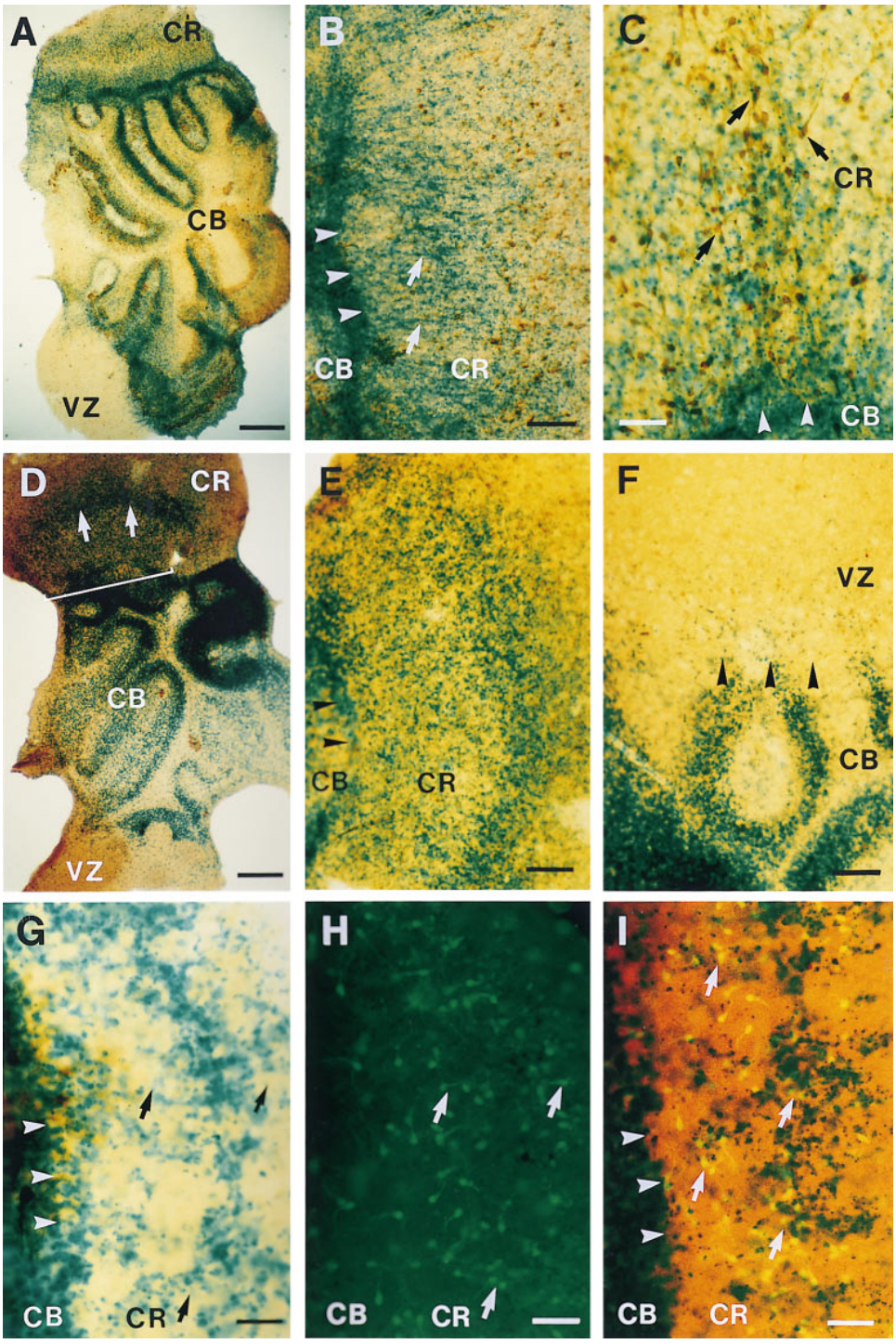

Figure 6. Effects of CR Cells on Bergmann Glia and on Migration of Granule Cells in Cerebellar Organotypic Cultures. Arrowheads Label the Neocortical–Cerebellar Interfaces

(A) Low power photomicrograph of a cerebellar slice (CB) from a Krox20–*lacZ*14 transgenic mouse cocultured with a CR cell–enriched slice (CR) and with a ventricular zone–containing slice (VZ) located at opposite sides. The triple coculture (8 DIV) was stained with Xgal to identify Bergmann glial cells and then immunoreacted for calbindin. Whereas blue Xgal+ Bergmann glia have penetrated and fill the CR cell–enriched explant (top), very few Bergmann glial cells penetrated and for shorter distances the VZ-enriched slice (bottom).

(B) High magnification of a Krox20–*lacZ*14 cerebellar slice (CB, left) cocultured with a CR cell–enriched explant (CR, right) demonstrating massive ingrowth of βgal-expressing Bergmann glia in the neocortical explant (8 DIV). In the area of the neocortical slice closest to the cerebellum, Xgal+ processes show radial orientation (arrows), whereas in deep areas, Xgal-staining appears more diffuse.

(C) CR cell-enriched slice (CR) cocultured with a Krox20–*lacZ*14 cerebellum (CB) stained with Xgal and immunolabeled with anti-calretinin antibodies to identify CR cells (8 DIV). Blue Xgal+ Bergmann glial cell processes are in close apposition with CR cells (arrows).

(D) Low power view of a cerebellar slice (CB) from a β2nZ3′1 transgenic mouse cocultured with a CR cell–enriched slice (CR) and with a

Table 1. Effects of CR Cell Explants on Radial Glia in Cerebellar Slices

Presence of RC2+ fibers in contact cocultures[a]				
	Number of Cultures			
	(−)	(+)	(++)	(+++)
CB+CR	0	1	4	12
CB+VZ	9	0	1	5
Ingrowth of βgal+ Bergmann fibers in contact cocultures[b]				
	Number of Cultures			
	(−)	(+)	(++)	(+++)
CB(Krox-20/*lac*Z14)+CR	0	0	9	18
CB(Krox-20/*lac*Z14)+VZ	10	7	1	0
Induction of RC2+ cells in semipermeable cocultures[c]				
	Number of Cultures			
	(−)	(+)	(++)	(+++)
CB+CR-wt	0	3	4	12
CB+ZV	13	2	0	0
CB+MB	4	0	0	0
CB+CR-*rl*/*rl*	0	0	1	5
CB+CR-*rl*/+	0	0	0	6

[a]Cerebellar slices (CB) were cocultured with CR cell-enriched slices (CR) or with slices containing the ventricular zone and the deep cortical layers (VZ) and immunostained with the RC2 antibody: (−), absence of RC2 immunoreactivity; (+), low numbers of RC2+ fibers (<10 fibers per 4×10^4 μm²); (++) moderate numbers of RC2+ fibers (>20 fibers per 4×10^4 μm²); (+++) higher numbers.

[b]Cerebellar slices from the Krox20/*lacZ*14 line were cocultured with CR or VZ explants and stained with Xgal: (−), virtual absence of Xgal+ Bergmann glia; (+), moderate ingrowth (<200 μm) of a low number of Xgal+ Bergmann glia; (++), deep (200–500 μm) massive ingrowth of Xgal+ Bergmann glia; (+++), very deep (>500 μm) massive ingrowth of Xgal+ Bergmann glia.

[c]Cerebellar slices were cocultured with CR cell-enriched slices from wild-type (CR-wt), *reeler* (CR-*rl*/*rl*) or heterozygous (CR-*rl*/+) embryos, or with VZ explants, separated by a semipermeable membrane. Other cultures were overlaid with a semipermeable membrane alone (MB). Cultures were immunostained with the RC2 antibody: (−), absence of RC2+ cells; (+), low induction of RC2+ cells (<10 cells per 4×10^4 μm²); (++), moderate induction of RC2+ cells (10–30 cells per 4×10^4 μm²); (+++), dramatic induction of RC2+ cells (>30 cells per 4×10^4 μm²).

Table 2. Effects of CR Cell Explants on Granule Cell Migration in Cerebellar Slices

	Number of cultures			
	(−)	(+)	(++)	(+++)
CB(β2nZ3′1)+CR	0	0	12	28
CB(β2nZ3′1)+VZ	2	17	3	0

Cerebellar slices (CB) from the β2nZ3′1 mouse line were cocultured with CR or VZ explants and stained with Xgal: (−), virtual absence of Xgal+ granule cells; (+), moderate ingrowth (<200 μm) of low numbers of Xgal+ granule cells (10–20 cells per 10^4 μm²); (++), deep (200–500 μm) massive ingrowth of Xgal+ granule cells (>100 cells per 10^4 μm²); (+++), very deep (>500 μm) massive ingrowth of granule cells (>100 cells per 10^4 μm²).

A Role for CR Cells in Directional Migration

Our experiments using the β2nZ3′1 line show that CR cells shift the direction of migrating granule cells that normally migrate toward the internal granule cell layer (Figure 7B). Moreover, granule cells migrated much deeper into the CR cell–enriched slice than those transversed in vivo in the cerebellum (Miale and Sidman, 1961). One possible explanation is that migrating neurons glide passively onto the Bergmann fibers that have grown into the cortical explant. In the living animal, however, migrating neurons riding along radial glia appear to migrate in specific directions. For instance, in the cerebellum, granule cells migrate from the apical glial end feet toward the cell body region of Bergmann glia, and in the neocortex, migrating neurons move toward the apical end feet in layer I. Furthermore, optical recording in living organotypic slices has shown that migration is largely unidirectional (Komuro and Rakic, 1995; Rivas and Hatten, 1995). Interestingly, directional migration appears to be lost in dissociated cell cultures, in which migrating neurons move backward and forward along radial glia (Edmondson and Hatten, 1987; Hatten, 1990; Gasser and Hatten, 1990), and to a lesser extent in the neocortical intermediate zone where neurons migrate tangentially along axon fascicles (O'Rourke et al., 1992, 1995). Taken together, these data suggest that migrating neurons can move in both directions along radial glia but that the potential for bidirectional migration is largely restricted in the living animal and in histotypically organized slices by tissue-dependent cues.

An alternative explanation for the present findings is that CR cells might exert a chemoattractive influence on migrating granule cells. Some neurotransmitters and growth factors have been found to exert chemotactic influences on immature neurons including neural progenitors and migrating neurons (Komuro and Rakic, 1993; Behar et al., 1994). A chemorepulsive factor derived from septum is involved in the direction of migrating neurons from the subventricular zone to the olfactory

ventricular zone–enriched slice (VZ). The culture was stained with Xgal to visualize cerebellar granule cells and then immunolabeled with an anti-calbindin antibody (10 DIV). Many blue βgal+ granule cells have migrated deep in the CR cell–enriched slice (top) where they concentrate in a band (arrows) at 400–500 μm from the cerebellar slice. Few Xgal+ granule cells have migrated into the VZ-containing slice (bottom). Boxed area is shown at higher magnification in (E).

(E) High magnification of the triple coculture in (D) shows massive penetration of blue Xgal+ granule cells in the CR cell–enriched slice.

(F) High power view of a β2nZ3′1 cerebellar (CB) slice cocultured with a ventricular zone–enriched slice (VZ) (10 DIV). Note the moderate migration of Xgal+ cerebellar neurons into the VZ-enriched slice.

(G and H) Pair of bright-field (G) and fluorescence (H) photomicrographs of the same field in a CR cell–enriched slice (CR) cultured with a β2nZ3′1 cerebellar slice (CB), showing migration of Xgal+ cerebellar granule cells in the CR cell–enriched explant (G) and survival of many CR cells identified by calretinin immunofluorescence (H) (8 DIV). Arrows labeling to same reference fields.

(I) Double-exposed photomicrograph (bright field and epifluorescence microscopy) of a CR cell–enriched slice (CR) cultured with a β2nZ3′1 cerebellar slice (CB) illustrating the close spatial relationships between the distribution of CR cells (arrows) identified with calretinin antibodies (yellow) and the clusters of blue Xgal+ granule cells (8 DIV). Scale bars = 250 μm, (A) and (D); 100 μm, (B), (E), and (F); and 50 μm (C), (G), (H), and (I).

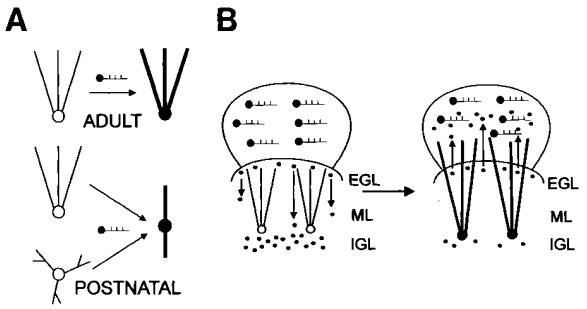

Figure 7. Summary Scheme on the Main Findings of the Present Study

(A) While transplantation of CR cells in the adult cerebellum leads to induction of RC2 immunoreactivity (bold) only in Bergmann glia (top), CR cells induce a RC2+ radial glia phenotype in Bergmann glia and in other astrocytes in the postnatal cerebellum (bottom). Moreover, postnatal cerebellar glial cells shift to an immature bipolar morphology typical of immature radial glia.

(B) CR cells in contact with the proliferative external granule cell layer (EGL) of postnatal cerebellar slices (left) lead to a massive ingrowth of Bergmann glial cell processes and cells into the CR cell–enriched explant (right). At the same time, postmitotic granule cells, which normally migrate toward the internal granule cell layer (IGL), now migrate also in the opposite direction, deep into the CR cell–enriched slice.

bulb (Hu and Rutishauser, 1996), suggesting that neuronal migration may be under the influence of both chemoattractive and -repulsive signals, similar to those regulating the navigation of axonal growth cones (Colamarino and Tessier-Lavigne, 1995a, 1995b; Messersmith et al., 1995). In fact, one such signal (netrin-1/UNC-6), may guide migration in nematodes and in the murine brain stem (Serafini et al., 1996; Wadsworth et al., 1996). Thus, although additional experiments are needed to ascertain whether CR cells exert chemoattraction on migrating cells, the dramatic alteration of migration reported here and the close spatial relationships between ectopic granule cells and CR cells (Figure 6I), together with the strategic disposition of CR cells in layer I, lead us to hypothesize that this is indeed the case.

Functions of CR Cells in Corticogenesis

The CR cells are generated early, before the onset of migration of the remaining cortical neurons, and largely disappear just after the period of migration. Previous studies of the *reeler* mutation have shown that CR cells are involved in neuronal migration (Goffinet, 1995; D'Arcangelo et al., 1995). The present data, together with complementary ablation studies (H. Supèr, J. A. D. R., A. Martínez, and E. S., unpublished data), provide evidence that the CR cells may be crucial in the regulation of the radial glia phenotype and suggest that they might also have a chemoattractive influence on migrating cells. The onset of CR cells during development coincides with the emergence of radial glia (Misson et al., 1991), suggesting that CR cells are involved not only in maintaining the radial glia but in inducing their differentiation in early corticogenesis. Thus, the first generated cortical neurons fulfill two functions that are essential for the ontogeny of the cerebral cortex: whereas subplate neurons and CR cells are involved in the guidance and reshaping of cortical afferents (Ghosh et al., 1990; Ghosh and Shatz, 1992; Allendoerfer and Shatz, 1994; Del Río et al., 1997), CR cells are also likely to organize the radial glial scaffold, which guarantees the "inside-out" order of migration and corticogenesis.

Experimental Procedures

Microdissection of Donor Tissue

Timed pregnant females of the OF1 strain (Iffa Credo, Lyon, France) were deeply anesthetized with ether. For the preparation of CR cell–enriched slices, the cerebral cortices of E15–E16 mouse embryos were excised and placed in phosphate-buffered saline with glucose (6 mg/ml). Small pieces of neocortex were embedded flat in melting (40°C) 3% agar with the marginal zone layer I side up. Agar blocks were cooled with ice and cut with a vibratome at 60–80 μm, tangential to the pial surface. Only the first vibratome section of each block, containing the marginal zone, was harvested and kept in ice-cold phosphate-buffered saline glucose. In some cases, tangential slices of the marginal zone were obtained from homozygous NSE–*lacZ* transgenic mouse embryos (Forss-Peters et al., 1990). To obtain vibratome slices enriched in cortical progenitors from the ventricular and subventricular zones, pieces of neocortex were embedded in agar with the ventricular surface side up and further processed in the same way. Most such vibratome slices were then used for transplantation or slice culturing. Other slices were fixed in 2% paraformaldehyde, embedded flat in gelatine, cryoprotected with sucrose, and cut transversally at 12 μm. Sections were Nissl stained or immunoreacted with calretinin antibodies (Swant Antibodies, Bellinzona, Switzerland) or the Rat–401 and RC2 mAbs (Hockfield and McKay, 1985; Edwards et al., 1990). In other cases, dissected embryonic brains were coronally sectioned with a tissue chopper at 300 μm. Small blocks containing all neocortical layers were isolated with tungsten microdissecting needles under an operating microscope (total cortex prepration). To obtain cortical progenitors and postmitotic neurons, with the exception of CR cells (total cortex minus CR cells), the marginal zone was removed from the 300 μm thick coronal slices using microdissecting needles.

Transplantation into Adult Cerebellum and Immunostaining

Embryonic cortical tissue obtained as above was grafted as solid grafts into young adult hosts (1- to 3-month-old) of the OF1 strain. After choral hydrate anesthesia (300 mg/kg body weight), mice were immobilized in a stereotaxic frame and transplanted as described elsewhere (Sotelo et al., 1994). Embryonic cortical tissue was drawn into a glass cannula, and the graft was deposited at variable depths within the host cerebellum. After recovery, mice were returned to their cages and perfused with 0.1 M phosphate-buffered 2% paraformaldehyde after several survival times (4–20 DAG). After dissection, the cerebella were postfixed in the same fixative for 2–4 hr, cryoprotected with sucrose, and cut on a freezing microtome at 40 μm in the sagittal plane. Parallel sections were incubated overnight with a rabbit anti-calretinin antibody (dilution 1:2000–1:4000; Swant Antibodies, Bellinzona, Switzerland) or with the RC2 mAb (dilution 1:5–1:10). Thereafter, sections were sequentially incubated with appropriate biotinylated secondary antibodies and the avidin–biotin peroxidase complex (ABC; Vector, Burlingame, CA). After developing peroxidase activity with 0.03% diaminobenzidine (DAB) and 0.005% hydrogen peroxide, sections were mounted on slides and coverslipped.

Sections from the cerebella grafted with tissue from NSE transgenic embryos were first incubated with 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (Xgal) as described elsewhere (Sotelo et al., 1994) to reveal βgal activity. Sections were then immunoreacted with the RC2 antibody as above. Four young adult homozygous mice of the Krox20–*lacZ*14 transgenic line (B6D strain; Sotelo et al., 1994) were grafted with CR cell–enriched slices. At 7 DAG, mice were perfused, and the cerebella were sectioned as above. Sections were immunostained with a rabbit anti-βgal antibody (1:1000; Cappel, Malvern, PA) and with the RC2 mAb (1:5). Primary

antibodies were visualized with fluorescein- and Texas Red–conjugated secondary antibodies.

Slice Culture Experiments

Cerebellar slice cultures were prepared from P5–P8 (P0, day of birth) cerebella, essentially as described (Stoppini et al., 1991; Del Río et al., 1996). Cerebellar cultures were from postnatal mice of the OF1 strain or from the Krox20–*lacZ*14 and β2nZ3′1 transgenic lines. Animals were anesthetized by hypothermia, their brains were aseptically removed, and the cerebella were excised under microscopic control. Tissue pieces were cut into sagittal slices (400 μm thick) using a tissue chopper and maintained in Gey's balanced salt solution supplemented with glucose (5 mg/ml) at 4°C. Then, cerebellar slices were placed onto 30 mm sterile membranes (Millicell-CM, Millipore, Bedford, MA). Cerebellar slices were cocultured with freshly prepared neocortical slices by positioning nearby both tissue pieces so that the neocortical slice was in contact with the external granular layer. Neocortical slices consisted of either CR cell–enriched slices, VZ-enriched slices, and cortex-minus-CR cell preparations. For the triple coculture experiments, in which several tissue combinations were assayed, two different neocortical slices were positioned at opposite sides of the cerebellar slice, and their position was recorded. Cultures were maintained in a humidified incubator at 36°C in 5% CO_2 for 7–14 days. The culture medium, composed of 50% basal medium (Eagle, BME), 25% Hank's balanced salt solution, 25% heat-inactivated horse serum, 1 mM glutamine, and glucose (5 mg/ml), was changed every 2–3 days. After fixation with 2% paraformaldehyde, slice cultures were removed from the membranes and immunostained with the RC2 mAb. Cocultures arising from transgenic mice were stained with Xgal for the visualization of βgal activity and then immunostained with a CaBP antiserum (dilution 1:8000; Spencer et al., 1976). To allow the identification of CR cells, cultures were subsequently processed for the visualization of calretinin using immunofluorescence. Other cultures from transgenic mice were immunolabeled with rabbit anti βgal antibodies and either parvalbumin (1:1000) or calretinin (1:1000) antibodies (Swant), followed by fluorochrome-coupled secondary antibodies.

For the diffusible test assays, cerebellar organotypic slices, mounted onto Millicell CM membranes, were overlaid by a semipermeable membrane (0.4 μm pore size; Biopore, Millipore). Neocortical vibratome slices were then positioned onto the semipermeable membrane, just above the cerebellar cultures, and their position was recorded. After 7–10 days, cerebellar cultures were fixed and immunoreacted with the RC2 mAb.

In another set of experiments, CR cell–enriched slices were obtained from E16 *reeler rl*^{orl}/*rl*^{orl} and *rl*^{orl}/+ embryos (Balb–C; Guenet, 1981). To identify homozygous and heterozygous embryos, the hippocampus and adjacent neocortex of each embryo was sectioned and Nissl stained, and the cytoarchitectonic pattern was recorded. CR cell–enriched slices from *reeler* embryos were used in experiments with semipermeable membranes as described above and in contact cocultures with cerebellar slices from heterozygous β2nZ3′1 transgenic mice.

Additional Material

Mouse embryos (E15–E16) were perfused with 4% paraformaldehyde, and their brains were excised. Vibratome sections (50 μm thick) were immunoreacted for calretinin as described elsewhere (Del Río et al., 1995). In addition, thick tangential cortical slices of perfused P0–P2 mice were immunostained for calretinin to allow the visualization of CR cells in vivo at ages comparable to that after transplantation or culturing.

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